Predictive Value of the Uterotrophic Assay for Genistein Carcinogenicity in the Neonatal Mouse: Relevance to Infants Consuming Soy-Based Formula

Markey et al. (*1*) recently questioned the sensitivity to estrogens of the rodent uterotrophic assay. However, an example of the biological validity of this assay was recently provided by studies on diethylstilbestrol (DES) and genistein by Newbold et al. (*2*). These investigators used the neonatal mouse uterotrophic assay to define estrogen-equivalent subcutaneous (sc) injection doses of these two chemicals: 1 µg/kg/day DES and 50 mg/kg/day genistein (a difference of 50,000-fold; Table 1). This estrogen-equivalent factor is identical to observations in similarly conducted weanling mouse uterotrophic assays of DES and genistein (Table 1) (*3*). Newbold et al. (*2*) also used the same dose levels to evaluate neonatal carcinogenicity of DES and genistein in the mouse uterus; they observed similar incidences of uterine adenocarcinomas with both chemicals in 18-month-old mice (Table 1). This confluence of findings in the neonatal and weanling mouse, and the similar carcinogenic potency of the two chemicals when evaluated at uterotrophic-equivalent dose levels, is encouraging for the utility of the rodent uterotrophic assay for predicting adverse effects in rodents. In particular, the fact that there is insufficient genistein in soy-based infant formula to trigger a uterotrophic response in rodents drinking the formula *ad libitum* indicates the probable absence of a carcinogenic hazard under normal conditions of use (*4*). It is of interest that the estrogen-equivalent dose levels of these two chemicals in estrogen receptor binding assays are separated by a factor of only 9,000 (*5*). The expansion to the 50,000 factor seen in the mouse assays is probably associated with disparities in target tissue dose due to differences in serum protein binding and excretion of the two chemicals *in vitro* and *in vivo*, differences that would need to be evaluated separately for humans.

Newbold et al. (*2*) suggested that the neonatal carcinogenicity of genistein should trigger a close examination of the potential hazard posed to infants by the consumption of soy-based infant formula, because genistein is the major isoflavone phytoestrogen formed from ingestion of soy products. The need for such a review was based on the assumption that 50 mg/kg/day genistein by sc injection, as used in the neonatal carcinogenicity bioassay, was representative of the levels of exposure to genistein experienced by infants fed soy formula (*2*). Given the potential societal interest in these new data (*2*), we suggest that the following facts should form a part of any review of the use of soy infant formula. First, genistein shows remarkable differences in route of administration in the uterotrophic assay, with the sc route being substantially more sensitive than the oral route (*3*). Thus, the use of the sc route by Newbold et al. (*2*) will inevitably have exaggerated the hazard posed to infants drinking soy formula. Second, the work of Setchell et al. (*6*), quoted by Newbold et al. (*2*) to support the relevance to infants of their dose of 50 mg/kg/day genistein, actually estimated that infants drinking soy formula are exposed to 6–11 mg/kg/day total isoflavones, a figure slightly higher than the 4.5 mg/kg/day estimated by the U.K. Food Standards Agency (*7*). In fact, oral administrations of genistein in this dose range (*6,7*) are inactive in the uterotrophic assay (*3,4,8*). Third, the major isoflavone in soy formula is not genistein, but rather its glycone conjugate, genistin (*6*). This conjugate is transformed into the estrogen genistein in the gut, a transformation attenuated in infants (*6*). Thus, it is tenuous to relate experimental data derived from the sc injection of genistein to the hazard posed to infants by drinking infant formula containing the glycone genistin. Fourth, the mouse chow used by Newbold et al. (*2*) (NIH-31) contained 46 µg genistein/g diet, which is similar to the 42 µg genistein/g in the RM1 diet (*9*) used by Ashby (*3*). Consequently, the control and test pups used in the carcinogenicity bioassay of genistein were potentially exposed to low levels of genistein *in utero* via the milk, and in their lifetime by the diet (a mouse weighing 25 g eating 4 g of diet a day would be exposed to ~7 mg/kg/day genistein, together with other isoflavones and their conjugates). The zero incidence of uterine adenocarcinomas in the control animals used by Newbold et al. (*2*) therefore suggests the existence of a threshold dose for the carcinogenicity of genistein, as observed in the uterotrophic assay (*3*). A final complication to the proposed safety review on infant formula is that uterotrophic activity and advanced sexual development is observed in rodents exposed to commercial infant formula via their drinking bottles (*4*). These effects were unrelated to the constituent phytoestrogens but were associated with centrally mediated nutritional influences leading to advanced puberty (*4,9*).

In conclusion, although the neonatal carcinogenicity of genistein is of significant scientific interest, the test protocol employed in the generation of those data render them of little value for purposes of infant risk assessment. In particular, the exposures to genistein experienced by the mouse neonates may have been several orders of magnitude higher than those experienced by infants drinking soy-based formula.

Table 1. Confluence of the neonatal and weanling mouse uterotrophic assay data and the neonatal mouse uterine carcinogenicity data for DES and genistein.

Period of dosing; assay end point (reference)	DES (1 µg/kg/day) [~0.002 µg/neonate]	Genistein (50 mg/kg/day) [~100 µg/neonate]
PND 1–5; uterotrophic assay at PND 5 (*2*)	190% uterine weight gain	202% uterine weight gain
PND 20–23; uterotrophic assay at PND 24 (*3*)	200% uterine weight gain	240% uterine weight gain
PND 1–5; incidence of uterine adenoma at 18 m (*2*)	31%	35%

All of the data compared involved sc injection of the test agents at the postnatal days (PND) shown. There were no uterine adenocarcinomas in the control mice (*2*).

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The Mouse Uterotrophic Assay: Other End Points

In their *EHP* article titled "The Mouse Uterotrophic Assay: A Reevaluation of Its Validity," Markey et al. (*1*) argue against using the uterotrophic assay as an end point for determining estrogenicity of synthetic chemicals. They conclude (*1*),

> The uterotrophic assay is of limited value in determining the estrogenicity of a suspected environmental estrogen because changes at the cellular level were observed at significantly lower doses than those at which a change in wet weight occurred.

In agreement with their findings, we have similar data which show that many cellular and biochemical end points in the uterus, such as epithelial cell height, cell number, gland number, and lactoferrin induction, are often more sensitive end points than wet weight increase. We have reported this information at international meetings and have shared our findings with the U.S. Environmental Protection Agency's Endocrine Disruptor Screening and Testing Advisory Committee. We have also compared the sensitivity of different end points for 10 compounds over a large dose range for each chemical (*2*). However, we disagree with the conclusion of Markey et al. (*1*) that the uterotrophic assay has limited value. Our increasing knowledge about the various actions of estrogens now makes it feasible to expand the uterotrophic assay to include information about different pathways and end points. Thus, we suggest that the uterotrophic assay be expanded to encompass additional measures to the standard wet weight data to increase its sensitivity. The objective of studying these end points when the uterotrophic assay is negative is to eliminate false negatives. Further, mechanistic information can be gained by developing a "blueprint" of responses for various estrogenic compounds. The mouse uterotrophic assay is valid: a reevaluation of the assay should assure that the assay is optimized so that meaningful and informative end points are included that cover a range of effects induced by the chemicals under study.

Two other points are worthy of mention. Numerous studies appearing in the literature report data from the uterotrophic bioassay as wet weight increase, when in fact they are reporting "blotted" uterine weight. The true wet weight of the uterus includes uterine tissue plus luminal fluid. Recording only "blotted" weight overlooks the significant role estrogens play in uterine water imbibition, an early marker of estrogen action. This is an important end point that again makes the assay more sensitive.

Another important point is that proliferating cell nuclear antigen (PCNA), or any other marker of uterine cell proliferation, may give erroneous results if measured after 3 days of continuous treatment with an estrogen, as described by Markey et al. (*1*). Estrogens are known to increase mitosis, but they also act to inhibit mitosis if the estrogen dose is too high or if it is sustained (Table 1). This may explain why the authors found no significant difference in the expression of PCNA in the luminal epithelium between treatment groups (control, estradiol, and bisphenol A), although other studies have shown increases in PCNA expression following treatment with these same compounds (*3*). Using time-course experiments in the immature mouse, we have determined that excellent PCNA labeling occurs 18 hr after an initial dose of estradiol or diethylstilbestrol (DES) (*2*). The time for maximum stimulation of mitosis varies with the pharmacokinetics of the compound being tested (*2*), but most chemicals, including known estrogens that we have tested thus far, showed slight or no increase in mitosis after 3 days of treatment. In fact, mitosis is inhibited by 3 days of treatment with DES (Table 1).

In summary, we agree with Markey et al. (*1*) that the current uterotrophic bioassay (consisting of only wet weight) is not sensitive, and we encourage a reevaluation of the assay in the framework of the information presented.

Table 1. PCNA-labeled uterine epithelial cells after treatment with DES or estradiol.

Treatment	Percent PCNA-labeled cells
Control	5.92 ± 0.79
DES 1×	31.81 ± 3.78*
Estradiol 1×	46.67 ± 3.68*
DES 3×	1.23 ± 1.23
Estradiol 3×	9.60 ± 0.84

Immature outbred CD-1 mice were administered 10 µg/kg DES or 500 µg/kg estradiol dissolved in corn oil by sc injection for 1 day or 3 days and sacrificed the morning following the last injection (*2*). These doses of DES and estradiol have been previously determined to cause maximum uterine wet weight increase (*4*). Uterine tissues were collected and PCNA was determined by previously described methods (*2,5*).
*p<0.05; statistically significant difference from control by ANOVA.

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The Rodent Uterotrophic Assay: Response to Ashby and Newbold et al.

The comments submitted by Ashby et al. and Newbold et al. reflect disagreement with our statement that the classical mouse uterotrophic assay is of limited use in assessing the estrogenicity of chemicals. We restate here that the uterotrophic assay is a relatively insensitive test for determining whether a chemical is estrogenic, for establishing no-observed-effect levels (NOELs), and ultimately for predicting the level of exposure at which adverse effects may occur. Ashby and colleagues cite a recent publication by Newbold et al. (*1*) as "an example of the biological validity of this [uterotrophic] assay." The study to which Ashby et al. refer shows that exposure of neonatal mice to daily injections from postnatal days 1–5 of either genistein (50 mg/kg/day) or DES (1 µg/kg/day) induces a uterotrophic response by the end of day 5 and uterine adenocarcinoma by 18 months of age. Ashby et al. interpret "this confluence of findings" as being "encouraging for the utility of the rodent uterotrophic assay for predicting adverse effects in rodents."

First, the study of Newbold et al. (*1*) does not conform to the classic use of the uterotrophic assay. This assay is characterized by a 3-day exposure of an adult ovariectomized or juvenile (prepubertal) rodent to a chemical, or in the case of the developmental uterotrophic assay, a 12-day exposure of a rodent from postnatal day (PND) 10 to PND 22 (*2*). Newbold et al. (*3*) cited Bern's study (*4*), which shows that the perinatal mouse is far more sensitive to the effects of estrogen exposure than the prepubertal or adult mouse, a widely held tenet in the field of endocrine disruption.

Second, by using a single dose of genistein and DES, the study of Newbold et al. (*1*) was not aimed at determining the minimal exposure that would cause an effect for

the end points of uterine wet weight and uterine adenocarcinoma at these ages. To emphasize this point, previous work from Newbold's group has demonstrated that exposure of neonatal mice to a 10-fold lower dose of genistein (5 mg/kg) resulted in the development of multi-oocyte follicles by 2 months of age (*5*), an effect that is likely to compromise fertility. Yet, a 3-day exposure of prepubertal mice (days 17–19) to < 50 mg/kg genistein was insufficient to induce a uterotrophic response (*6*). Thus, for the parameter of multi-oocyte follicles, the uterotrophic assay is not predictive.

Third, induction of adenocarcinoma is the most extreme of biological end points to consider, particularly in a field that is attempting to tease out the subtleties of biological perturbations due to chemical exposure in populations characterized by variation. Again, to emphasize this point, Newbold (*7*) demonstrated that exposure of CD-1 mice to 100 μg/kg DES on gestational days 9–16 induced uterine adenocarcinoma; yet a dose of DES that was 10,000-fold lower (0.01 μg/kg) resulted in reduced reproductive capacity. By using the uterotrophic assay to establish the estrogenicity of bisphenol A (BPA), it can be concluded that exposure to a dose < 100 mg/kg/day is not detrimental to CD-1 mice. However, exposure of the same strain of mice during gestational days 9–20 to doses of BPA 4,000 times lower (25 μg/kg/day) induced profound proliferative changes in the lobuloalveolar network of the adult mammary gland (*8*). We also observed the persistence of epithelial structures that have been shown to be sites of neoplasia in rodents and humans, and an alteration in DNA synthesis between the epithelium and stroma, which is the substratum of neoplasia in this tissue (*8*).

Ashby's statement that there is "insufficient genistein in soy-based infant formula to trigger a uterotrophic response in rodents" which "indicates the probable absence of a carcinogenic hazard" leads him to conclude that the dose of genistein found in infant formula is not of biological concern. This rationalization clearly demonstrates the dangerous misuse of the uterotrophic assay that we pointed out in our paper in *Environmental Health Perspectives* (*9*).

Newbold et al. support our findings that "many cellular and biochemical end points in the uterus … are often more sensitive end points than wet weight increase," yet they disagree with our purported conclusion that the uterotrophic assay has limited value. We concluded that "the uterotrophic assay is of limited value in assessing the estrogenicity of a suspected environmental estrogen" (*9*), and we maintain this position on the basis of our work and that of others. Research by Newbold and colleagues clearly demonstrates this phenomenon (*3*). This group's study showed that exposure of prepubertal mice (days 17–19) to a variety of chemicals induced a uterotrophic response in all cases except with endosulfan and kepone. In spite of an observed increase in the number of uterine glands and PCNA labeling, these two chemicals were not able to induce a uterotrophic effect at any dose, even at those doses that were toxic to the animal (endosulfan). Although this work provides a seminal contribution to the field of endocrine disruption, it cannot claim to have "compared the sensitivity of different end points, for 10 compounds, over a large dose range for each chemical." Newbold et al. (*3*) described a dose–response curve for 10 chemicals, using the uterotrophic assay as the end point. Then, they tested the change in various cellular and biochemical end points at the dose that induced the maximal uterotrophic response.

We agree with the statement of Newbold et al. that the mouse uterotrophic assay should be "optimized so that meaningful and informative end points are included that cover a range of effects induced by the chemical under study." However, we propose that this modified assay be renamed so as not to be confused with the classical mouse uterotrophic assay, which is simply a measure of the increase in uterine wet weight after exposure to a chemical. In redefining the assay, we should consider the statement by Roy Hertz (*10*) who suggested that

> the sine qua non of estrogenic activity remains the mitotic stimulation of the tissues of the female genital tract. A substance which can directly elicit this response is an estrogen; one that cannot do this is not an estrogen.

In regard to the issue of measuring uterine wet weight versus "blotted" weight that was raised by Newbold et al. in their letter, we believe that a universally accepted technique should be used by all researchers to accommodate a valid comparison of data. In our study, we chose to define uterine weight as "blotted" because we believe that it is closer to the ideal of Hertz (*10*), as outlined above, which asserts that mitotic activity is the best index of an estrogenic effect.

In conclusion, we maintain our original position that the uterotrophic assay is not sufficiently sensitive to be used as a tool to determine whether or not a chemical is estrogenic or to establish NOELs. In the absence of other more sensitive end points, relying on the uterotrophic assay alone to make statements that exposure to certain estrogenic chemicals is not of biological concern, as appears to be the modus operandi of some researchers in the field, is hazardous to the health of all species.

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Are All Cigarettes Equal?

Ezzati and Kamen (*1*) correctly add an increment to the estimated personal exposure of smokers in their Kenyan cohort to account for the mass of particulate matter (PM) that is inhaled directly from the mainstream smoke (*2*). However, the authors added only 1,000 μg/m^3 to the smoker's personal total PM exposure. Kenyan smokers can choose between smoking Kiraiku (home processed, hand-rolled tobacco) and commercial filtered and nonfiltered cigarettes (*3*). I expect that smoking of Kiraiku and nonfiltered commercial cigarettes may well deliver much more than 17 mg of tar per cigarette (*4*). Thus, if the authors' cigarette-smoking subjects actually smoked even one such cigarette per day, they would have an increment to their personal exposure that would be much larger than 1 mg/m^3.

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Are All Cigarettes Equal?: Response

Mage raises the issue of exposure to particulate matter from smoking in our study of indoor smoke and acute respiratory infections in Kenya (*1*), but the points he raises, although correct, are not applicable in this context. In our study area, smoking was very uncommon. Only a small subset of the group smoked cigarettes (a total of 13 in our study group). Those who did smoke did so very infrequently, often not even on a daily basis, and they often shared their cigarettes because of the cost of cigarettes and because the most common habit in the area is chewing the leaves of the Mirraa plant. Although there are different types of cigarettes and tobacco available in Kenya, we only encountered commercially manufactured cigarettes in our study.

We added 1 mg/m^3 to the personal total PM exposure for smokers. We obtained this value by assuming a PM_{10} concentration of 400 mg/m^3 and approximately 4 min of active inhalation. In addition to being a source of particulate matter, smoking was considered as an independent factor in Tables 4 and 5 of our paper (*1*) to estimate its independent contribution to disease, a more informative source of information. By considering cigarette smoke as a source of PM, we acknowledged this parallel between the two pollutants (while emphasizing the differences in their other properties such as carcinogenesis).

With the uncertainties in assessment of exposure to pollution from cooking and using wide exposure categories, the small number of smokers, and multiple analyses approaches, our findings are not sensitive to the exact level of exposure to PM from smoking.

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Re: Risk Assessment of Internal Cancers from Arsenic in Drinking Water

Arsenic in drinking water was the first environmental health problem to be recognized. It has been regulated for 100 years, first by the British Royal Commission on Arsenic Poisoning of 1903 (*1*), then by individual states in the United States, by the U.S. Department of Health in 1942, and since 1970, by the U.S. Environmental Protection Agency (U.S. EPA) (*2*). The scientific basis of all arsenic standards, including the present 50 µg/L drinking water standard, is the toxic threshold concentration of around 250 µg/L (*1*). Arsenic-related skin cancer has that same threshold (*2*). Morales et al. (*3*) should have acknowledged the contradiction of their bladder cancer interpretation with the much stronger skin cancer evidence.

The U.S. EPA decided to regulate chemicals with a procedure called "Risk Assessment" that extrapolates risk below the threshold to a point of $1/10^{-6}$ risk as the regulatory target, or roughly 10,000 times below the threshold concentration. The 50 µg/L standard already is as strict as is feasible. The U.S. EPA did not apply risk assessment to arsenic for 30 years and continued to use the 50 µg/L standard. Now, in a controversial proposal, the U.S. EPA proposes to lower the standard 5-fold, using as official justification (*4*) the risk assessment by Morales et al. (*3*). The authors (*3*) state that

> … our analysis suggests that the current standard of 50 µg/L is associated with a substantial increased risk of cancer and is not sufficiently protective of human health.

The evidence presented by Morales et al. (*3*) in Table 5 of their paper can be interpreted as showing no increased risk in the 0–400 µg/L concentration range. Numbers of cases, with the standardized mortality ratio (SMR; shown in parentheses) for the ranges 0–50, 50–100, 100–200, 200–300, and 300–400 are 26 (10.0), 12 (4.2), 12 (10.5), 8 (7.7), and 6 (7.5), respectively, for bladder cancer, and 30 (1.6), 31 (1.4), 21 (2.4), 24 (3.1), and 12 (2.0), respectively, for lung cancer. Bladder cancer is uniformly elevated relative to the control population, but neither the raw case numbers nor the associated mortality ratios show a positive trend in the 0–400 µg/L range. The authors (*3*) admit that the "computed SMRs display a large amount of noise." A better interpretation for the data would be that there is an unexplained increase in bladder cancer. Above 600 µg/L, both bladder and lung cancer are positively correlated with arsenic, but may be confounded by smoking.

Regarding the threshold, Morales et al. (*3*) used two models to estimate the effective level of 1/100 risk (ED_{01}); values for male bladder cancer were 395 and 351 µg/L. The ED_{01} is roughly identical with the limit and the threshold of real risk. Both estimates are supported by the data and are in line with the Royal Commission safe level near 250 µg/L (*1*) and my interpretation that the threshold for bladder and lung cancer is > 400 µg/L.

Scientists have a special obligation if their interpretations are used by governments for regulation. That obligation requires clarification of the statement by Morales et al. (*3*) that "… the current standard of 50 µg/L is associated with a substantial increased risk of cancer" (p. 655). That statement is not supported by the data. It should also be kept in mind that two previous advisory panels on arsenic recommended that risk should not be extrapolated for arsenic (*5*). The most recent of these reports states that "[the U.S.] EPA has not requested, nor has the subcommittee endeavored to provide, a formal risk assessment for arsenic in drinking water" (*6*; p. 253).

The U.S. EPA has formally adopted the 10 µg/L arsenic standard since this letter was submitted (7).

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